1211

Effect of Freeze-Drying on Some Enzyme Systems of *Serratia marcescens*[1]

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Freeze-drying and subsequent rehydration subject bacteria to conditions that may induce injury. In some cases the permeability of the cell membrane may be altered so that substrates not utilized by the intact cell are readily attacked (Hestrin and Lindegren, 1950; Campbell and Stokes, 1951). However, the injury may result in the destruction of one or more sensitive enzymes, or factors necessary for enzyme activity. It has been reported that a freeze-dried preparation of *Serratia marcescens* dissimilates glucose in a two-step pattern instead of the steady rate observed with fresh cells (Wasserman *et al.*, 1956).

The studies herein reported suggest that changes which take place during freeze-drying, storage, or reconstitution, or a combination of these, may result in the destruction or inactivation of an enzyme or enzymes involved in the oxidation of 2-ketogluconic acid by *S. marcescens*.

Materials and Methods

Three freeze-dried preparations of *S. marcescens* were obtained from the Northern Utilization Research and Development Division, Agricultural Research Service, Peoria, Illinois. The cells for each preparation were grown in a medium of the same composition, concentrated, and combined with a drying stabilizer, quick-frozen and vacuum-dried under approximately the same conditions. At the time of testing, the percentages of viable cells present in vacuum sealed tubes of preparations 1, 2, and 3[3] were 70, 70, and 29, respectively. A loss of 30 per cent of the viable cells had occurred in preparation 2 during 18 months' storage over Drierite[4] at −18 C, whereas the cells of preparation 3 decreased from an initial 55 per cent drying recovery to 29 per cent after 10 months' storage at −18 C. Preparation 1 had been dried 2 weeks before the study began.

Warburg experiments were carried out by conventional manometric techniques, using glucose or 2-ketogluconic acid as substrate. Each flask contained 0.5 ml of 0.05 M Tris buffer [tris(hydroxymethyl)amino methane] pH 7.2; 100 μg of inorganic phosphorus; 0.5 ml of cell suspension (containing 4.0 mg dry weight); 2.5 μmoles of appropriate substrate; and water to 2.0 ml. Sodium arsenite, 0.1 ml of 0.1 M solution, was added as indicated in the text. Total gas exchange values were corrected for endogenous metabolism.

Calcium 2-ketogluconic acid was obtained through the courtesy of Dr. F. H. Stodola, of the Northern Utilization Research and Development Division, Agricultural Research Service. 2-Ketogluconic acid (2-KG) was determined colorimetrically by the method of Lanning and Cohen (1951), and chromatographically by the method of DeLey (1955).

Results

The oxidation of glucose by the three dried preparations of *S. marcescens* is shown in figure 1*A*. Preparations 1 and 2 oxidized glucose at a steady rate, although the rate of oxidation by no. 2 was somewhat slower than

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[3] Preparations 1, 2, and 3 refer to the freeze-dried *S. marcescens* preparations D-84-1, D-8, and D-37-4, respectively.

[4] W. A. Hammond Drierite Co., Xenia, Ohio.

that by no. 1. Preparation 3 oxidized glucose to almost the same extent, but in two steps, with a leveling off between the steps at approximately 1.3 moles of oxygen consumed per mole of glucose. Chromatographic analysis of the metabolite solution at the level of the first step revealed an accumulation of 2-KG.

With 2-KG substrate (figure 1*B*), immediate oxidation occurred only with preparation 1. There was a lag

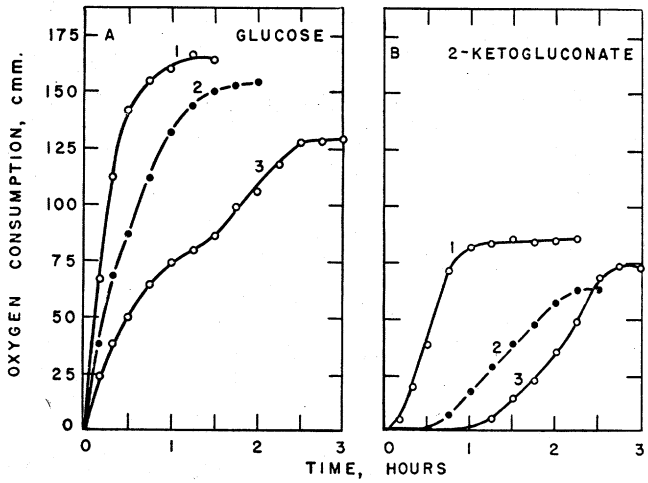


Figure. 1 Oxidative patterns of freeze-dried preparations of *Serratia marcescens* in the presence of glucose and 2-ketogluconate.

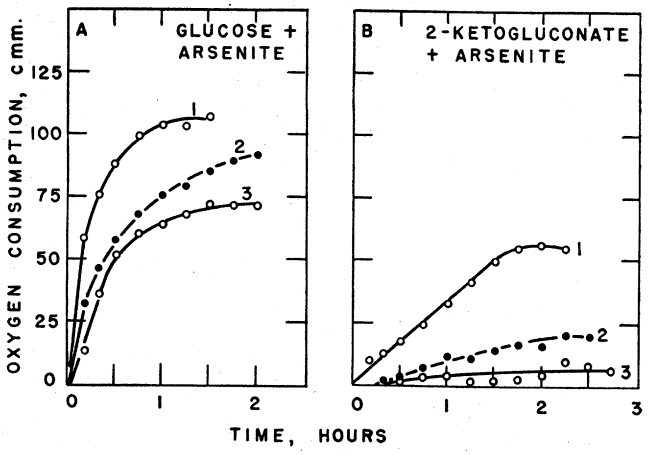


Figure 2. Oxidative patterns of freeze-dried preparations of *Serratia marcescens* in the presence of glucose and 2-ketogluconate, plus sodium arsenite.

TABLE 1

Carbon dioxide production from glucose and 2-ketogluconate by Serratia marcescens in the presence and absence of 0.005 M sodium arsenite

Preparation	μMoles of CO_2 per μMole of Substrate			
	Glucose	Glucose + arsenite	2-Ketogluconic acid	2-Ketogluconic acid + arsenite
1	3.0	2.1	2.2	2.2
2	2.8	1.4	2.0	0.2
3	2.5	0.3	2.0	0.3

period of about a half-hour before preparation 2 started to oxidize 2-KG, and approximately 1 hr before preparation 3 began to consume oxygen. Analysis of the supernatant during the lag period of the oxidation by preparation 3 indicated that all of the original 2-KG added could be accounted for as 2-KG-reactive material by the method of Lanning and Cohen (1951); thus extensive nonoxidative alterations in the 2-KG molecule apparently had not occurred.

Further emphasis of the differences among the dried cell preparations was obtained when 0.005 M sodium arsenite was added to the medium. Glucose oxidation (figure 2*A*) occurred with an uptake of 2 moles of oxygen per mole of glucose with preparation 1, and an uptake of 1.7 moles with preparation 2. However, the rate of oxygen consumption of preparation 2 in the presence of arsenite was considerably slower than that of preparation 1. The oxidation of glucose by preparation 3 was halted by the inhibitor at the first step of the oxidation (that is, at approximately 1.3 moles of oxygen per mole of glucose).

The oxidation of 2-KG by preparations 2 and 3 in the presence of arsenite (figure 2*B*) was almost completely inhibited, but with preparation 1 there was a decrease of only 1 mole of oxygen per mole of substrate.

Carbon dioxide evolution (table 1) similarly reflected the differences in the enzymatic composition among the cell preparations and the effect of arsenite on the metabolism of glucose and 2-KG. Without arsenite, the CO_2 formed from glucose was approximately the same for all three preparations. In the presence of the inhibitor, CO_2 evolution by preparation 1 was inhibited 33 per cent, preparation 2 was inhibited 50 per cent, and evolution of CO_2 by preparation 3 was almost completely inhibited. With 2-KG as substrate, about the same quantity of CO_2 was formed by all three preparations in the absence of arsenite. There was no effect of the inhibitor on CO_2 formation by the cells of preparation 1, but gas evolution by the other two preparations was almost completely inhibited.

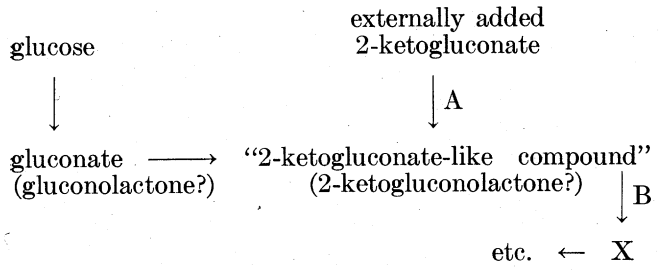
Discussion

The oxidative patterns of glucose and 2-ketogluconate dissimilation by three *S. marcescens* preparations, grown and freeze-dried presumably in a comparable manner, appears to indicate that at least one, and possibly two, enzymes were inactivated in drying or restoring the cells. The pattern of oxidation of the two substrates by preparation 1 was similar to that previously observed with freshly grown cell preparations. Preparation 1, therefore, may be considered to possess the normal patterns of oxidation. Although preparation 2 was able to oxidize glucose to approximately the same level as preparation 1, the decrease in rate indicates some impairment in enzymatic function. This preparation also required a lag period prior to 2-KG oxidation.

Preparation 3 oxidized glucose in two stages, and 2-KG only after a long initial lag period.

The lag periods required for 2-KG oxidation by preparations 2 and 3 probably indicate a period of synthesis required for the formation of the enzyme (or an activation factor) that can initiate the dissimilation of this substrate. Since this enzyme was active in preparation 1 it may be assumed that its inactivation occurred during the treatment of the other cell preparations.

Further enzyme loss is shown by preparation 3 in the oxidation of glucose; that is, after approximately 1.3 moles of oxygen per mole of glucose is taken up, the rate of oxygen consumption decreases and a compound reacting like 2-KG accumulates. This leads to the supposition that an enzyme converting 2-KG to the next compound in the metabolic chain has also been inactivated. However, this enzyme is present in preparation 2, which is capable of oxidizing glucose to completion without interruption. It is postulated, therefore, that the oxidation of glucose and 2-KG may proceed by the following pathway:



Thus, preparations 2 and 3 both lack enzyme A, which converts externally added 2-KG to a "2-KG-like compound," and preparation 3 also lacks enzyme B for further dissimilating the 2-KG-like compound. The necessity of activating two enzymes would also explain the longer lag period required for the oxidation of 2-KG by preparation 3. The nature of the "2-ketogluconate-like compound" is not known. However, Brodie and Lipmann (1955) and Cori and Lipmann (1952) have shown that glucose is oxidized to gluconolactone, which is hydrolyzed, phosphorylated, and oxidized to 2-keto-6-phosphogluconate. Although it is possible that the postulated intermediate is a phosphorylated 2-KG, chromatographic analysis has not revealed phosphorylated 2-KG compounds (Wasserman *et al.*, 1956). As an alternative, it is suggested that gluconolactone is oxidized in the lactone form to yield 2-ketogluconolactone, which is then decarboxylated to CO_2 and pentose.

Sodium arsenite is a classical inhibitor of pyruvic acid oxidation due to the reaction of the arsenite with the coenzyme, lipoic acid (Gunsalus, 1953). Thus, the inhibition of other oxidative decarboxylations (as in the further degradation of 2-KG) could imply the involvement of lipoic acid as a coenzyme. However, since 2-KG decarboxylation by preparation 1 continues uninhibited in the presence of arsenite, it is doubtful that the inhibitor prevents 2-KG dissimilation by this mechanism. It appears, therefore, that sodium arsenite may act by inhibiting enzyme (or enzyme activator) formation in preparations 2 and 3. Bernheim (1954) has shown that arsenite inhibited, to the extent of 50 per cent, the adaptive formation of benzoic acid oxidase by *Pseudomonas aeruginosa*.

The prolonged storage of two of the dried cell preparations may be a contributing factor in the decrease of the enzymatic activity, but if so, this factor should be of secondary importance. Theoretically, a cell dried without injury, and stored under ideal conditions, should rehydrate to its original state. However, cells injured during freezing or drying, even if stored under perfect conditions, could gradually deteriorate, or could be killed by improper rehydration. Thus preparation 3, with a 45 per cent loss in viable cells immediately following dehydration, and a further 46 per cent loss during 10 months of storage, was apparently seriously injured during the preparative process. Under these conditions the viable cells were still able to oxidize the substrates, although in an abnormal manner. Arsenite, in concentrations not harmful to normal cells, sharply restricted the enzymatic activities of these injured cells. Preparation 2 was frozen and dried with apparently very little injury to the cells, since 100 per cent recovery of viability was obtained immediately following dehydration, and only 30 per cent of the cells became nonviable following 18 months of storage. However, an injury must have been inflicted on the cells, since their enzymatic activity differed from that of normal cells. Preparation 1, dried shortly before use, had a recovery of 70 per cent of viable cells, and showed an enzymatic pattern similar to that of fresh cells. It was not possible to follow these cells in storage to determine whether any injury to the remaining viable cells would develop or whether the cell injuries that accounted for the initial deaths were the only ones sustained.

Thus at the present time it is not possible to definitely rule out storage as contributing to the enzymatic defects noted in this study, but it is believed the primary injury was sustained prior to storage.

Summary

A study of the glucose and 2-ketogluconate oxidations by three freeze-dried preparations of *Serratia marcescens* reveals differences in enzymatic composition that could occur as a result of freeze-drying or rehydration. The enzyme(s) involved in 2-ketogluconate oxidation, inactivated in two of the three preparations, could be reactivated during a lag period in the presence of the substrate. The relationship of the enzyme(s) to 2-ketogluconate oxidation, and the action of sodium arsenite, is discussed.

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